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TISSUE CULTURES OF CIRRIPEDS

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The *in vitro* techniques of explantation have been used in two different areas of research. For cell cultures the aim is to to obtain cell outgrowth from an explant and multiplication of the outgrown cells. Continuous cell lines may eventually be obtained. In organ cultures, on the other hand, the goal is to maintain the structural and functional integrity of the explant. Cell outgrowth should be avoided in organ culture, since the escape of cells disorganizes the explant.

Organ cultures have been obtained from malacostracan crustaceans and used in developmental, physiological, and endocrinological studies (Gomot, 1972). Most successful are perhaps the experiments by Berreur-Bonnenfant (1972) on the endocrine activity of the androgen gland in organ cultures of Amphipoda and Isopoda. Cell outgrowth has been reported from explants of decapod crustaceans (Peponnet and Quiot, 1971). Long term cell cultures have been obtained from tissues of the crayfish *Astacus pallipes* and the crab *Pachygrapsus marmoratus* (Vago and Quiot, 1969). Proliferating cells producing large surface spreads were obtained for four months in these cultures. Continous cell lines, however, have not been established from crustaceans.

In Cirripedia neither cell nor organ cultures have been reported. The number of unsolved problems concerning the physiology, development, and endocrinology of cirripeds calls for the development of *in vitro* culture techniques for this group. The present paper describes experiments which aim at establishing techniques and culture media suitable for tissue cultures in cirripeds.

Materials and Methods

Materials

Specimens of the acorn barnacle *Balanus amphitrite* were collected on the pilings of the dock at Duke University Marine Laboratory, Beaufort, North Carolina, during the months of April to August 1973. Adult animals in the size range of 10 to 15 mm in basal diameter and preferably with a flat basis, were used. The animals were brushed clean and kept in areated aquaria at $23 \pm 1°$ C and a sea water salinity of 30‰. At least one week of acclimation was allowed between collection and the experiments. During this period of time animals which had been injured during the collecting process could be removed. The animals were fed newly hatched *Artemia* nauplii (Metaframe, San Francisco Bay Brand) every second day. The water was changed weekly and the animals were then cleaned by light brushing. The animals were maintained for months in a good condition as judged by their feeding and molting activity.

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TABLE I

Culture media tested on barnacle tissues. Additions to the barnacle saline of Hoyle and Smith (1963) are listed in percent of the final media, except for Minimum Medium Eagle and Medium 199 where percentages refer to per cent of the concentrations recommended for vertebrate cells.

Medium	Glucose %	Bovine embryo extract %	Yeastolate %	Serum %	Minimum Medium Eagle %	Medium 199 %
I	0	0	0	0	0	0
II	0.1	0	0	0	0	0
III	0.1	1	0	0	0	0
IV	0.1	1	0.1	0	0	0
V	0	1	0.1	0	0	0
VI	0.1	0	0.1	0	0	0
VII	0.1	1	0.1	*Callinectes*, 10	0	0
VIII	0.1	1	0	*Callinectes*, 10	0	0
IX	0.1	1	0	Bovine, 10	0	0
X	0.1	1	0	*Callinectes*, 10 Bovine, 5	0	0
XI	0.1	1	0	*Callinectes*, 10 Bovine, 10	0	0
XII	0.1	0	0	Chicken, 10	0	0
XIII	0.1	0	0	Bovine, 10 Chicken, 5	0	0
XIV	0	1	0.1	0	10	0
XV	0.1	1	0	0	0	10
XVI	0.1	1	0	*Callinectes*, 10	0	10
XVII	0.1	1	0	*Callinectes*, 10 Chicken, 5	0	10
XVIII	0.1	1	0	*Callinectes*, 10 Chicken, 5	0	100

Tissue sampling

Before dissection animals were carefully cleaned and placed for 16 to 20 hours in a hood with the basal membrane pointing towards an utraviolet lamp (30 W) at a distance of 15 cm. On dissection the basal one third of the animal was dipped into 5% sodium hypochlorite for 2 seconds, rinsed in absolute ethanol for 5 seconds, and wiped with sterile cotton. The animal was mounted upside down under a low power microscope and the calcareous basis was carefully removed. Pieces of 1 to 2 mm^3 of mantle tissue with ovarioles and cement glands were sterile dissected out. One of the pieces was immediately fixed in Carnoy (3 ethanol: 1 acetic acid) and the other pieces were used for the cultures. Animals were discarded when the inner mantle membrane was ruptured during the dissection since cultures from these animals were inevitably contaminated.

Culture technique

Pieces of mantle tissue were transferred to Falcon plastic tissue culture flasks (25 cm^2). Each animal gave an amount of tissue sufficient for 2 to 4 flasks each with 4 to 6 explants. The explants were allowed to adhere to the bottom surface

of the flask for 30 to 60 seconds before 2 ml of culture medium were applied. About half of the explants remained attached while the others were free floating in the medium. The flasks were immediately gassed with a mixture of 5% CO_2 in air, capped, and incubated at 26° C in darkness. The medium was kept at a pH of 7.3 by gassing with the mixture of CO_2 in air when necessary. Phenol red was used as a pH indicator. The culture medium was changed every fourth day. The cultures were left undisturbed for the first 48 hours, whereafter the cultures were conrolled daily in a Zeiss inverted phase contrast microscope. The amount of cells migrating from the explants, and the quality and multiplication of these cells were studied. Contaminated cultures were discarded. Free floating explants were removed at certain intervals and fixed in Carnoy for histochemical studies. In some cultures explants attached to the plastic surface were removed and fixed to study the origin of the migrated cells.

Culture media

The tested culture media were based upon the barnacle saline of Hoyle and Smyth (1963) : 466 mM NaCl, 8 mM KCl, 20 mM $CaCl_2$, 12 mM $MgCl_2$, 10 mM $NaHCO_3$. The saline without $NaHCO_3$ was autoclaved (30 min at 18 psi). The $NaHCO_3$, prepared in a 5.6% solution and sterilized by filtration (millipore filter, pore diameter 0.22 μ), was added to the saline just before use. The tested culture media were prepared of barnacle saline with one or more additions as shown in Table I. Each medium was tested in 2 to 5 cultures. D-glucose (Sigma) was made up in a 20% solution and autoclaved. Dessicated TC Bovine Embryo Extract EE_{100} (Difco 5396), TC Yeastolate (Difco 5577), and TC Chicken Serum (Difco 5553–72) were resuspended in sterile, distilled and deionized water and stored at –20° C. TC Bovine Serum, ultrafiltrate (Difco 5473–72) was stored at –20° C. TC Minimum Medium Eagle, dried (Difco 0675) and TC Medium 199, dried (Difco 5701) were added directly to the saline in a concentration of 10 or 100 per cent of that recommended for vertebrate cells, and the solutions were immediately sterilized by filtration. Hemolymph of the blue crab, *Callinectes sapidus*, was sampled from the sinus in the merus of the chelipeds in adult, interecdysial (Passano, 1960) animals. Prior to bleeding the crabs were acclimated for 2 to 6 days to sea water of 30‰. The hemolymph was immediately heated to 60° C for 5 minutes to deactivate polyphenol oxidase (Wyatt, 1956). Hemolymph from 20 to 30 crabs was pooled and centrifuged at 31,000 × g for 20 minutes at 4° C in a Sorvall RC2-B centrifuge. The supernatant was filtered through a Millipore filter of pore diameter 0.65 μ with a prefilter, followed by sterile filtration with a prefilter. The filtrate was stored at –20° C. Penicillin (Sigma, Pen-na Benzylpenicillin Sodium Salt) and streptomycin (Sigma, Streptomycin Sulphate) were added to the culture media in concentrations of 100 I.U. per ml just before use.

Histology

The pieces of mantle tissue and cultured explants were fixed in Carnoy for 2 hours, washed twice in absolute ethanol, embedded in Paraplast (Fisher Scientific), and sectioned 5 or 8 μ. The sections were stained routinely with the Mallory-Heidenhain Azan Stain (Koneff, 1938) and with the Feulgen technique

(Pearse, 1968) with Fast green as a counter stain. For histochemical studies the following methods were used: general proteins by Mercuric brom phenol blue after Bonhag (Pearse, 1968); tyrosine containing proteins by the Millon reaction, Baker modification (Pearse, 1968); protein bound SH-groups by the DDD reaction (Barnett and Seligman, 1952) with maleimide block and iodoacetate block (Pearse, 1968) as controls; protein bound SS- and SH-groups by reaction with thioglycollic acid (Pearse, 1968) followed by the DDD reaction (Barnett and Seligman, 1952); RNA and DNA by the Methyl-green-pyronin Y method (Kurnick, 1955) with hydrolysis of RNA by ribonuclease as control; acid mucopolysaccharides and RNA by Toluidine blue O (Pearse, 1968) with ribonuclease as control. Photomicrography was made with a Zeiss Photomicroscope II.

Cultures of other species

Additional cultures were set up with tissues of two other balanids, *B. eburneus* and *B. improvisus*. Specimens of *B. improvisus* (13 to 15 mm in basal diameter) were collected in April 1973 on dead submerged tree trunks at Cherry Branch, Neuse River, North Carolina, and *B. eburneus* (15 to 25 mm in basal diameter) were collected on the laboratory dock in August 1973. The specimens of *B. eburneus* were molt-staged according to the method of Davis, Fyhn and Fyhn (1973) using rami of the sixth pair of cirri. Otherwise the treatment of the animals and the culturing procedure were as described for *B. amphitrite*. Twenty-one cultures were obtained with tissues of *B. eburneus* and 7 cultures of *B. improvisus*.

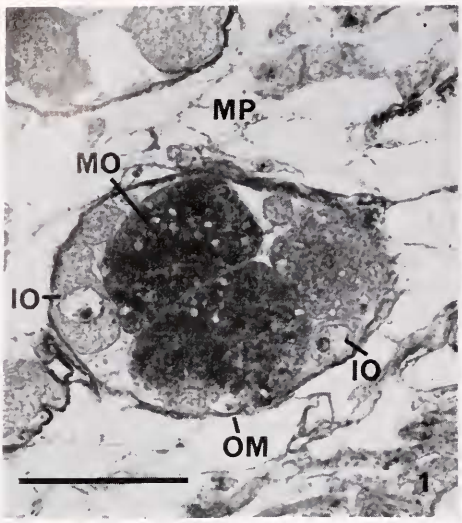
Results

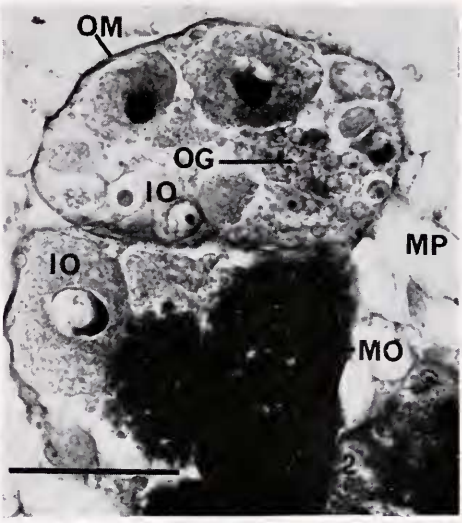
Organ cultures

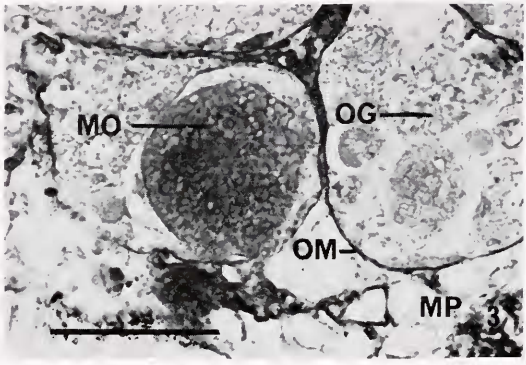
Quality of culture media was determined by comparing the histology of free floating explants with the histology of uncultured tissue of the same animals (the *in vivo* condition). The explants were cultured for three and seven days. Three different types of ovarian cells were used in the comparison: mature oocytes, immature oocytes, and oogonia.

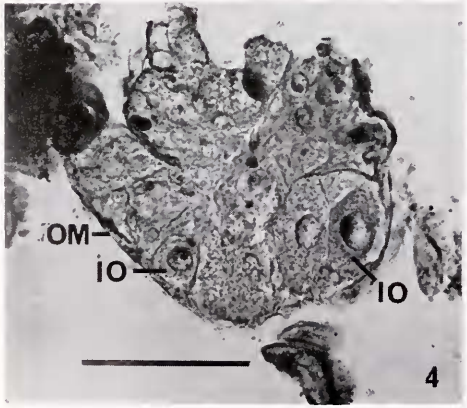
In the *in vivo* condition (Fig. 1) the mature oocytes of *Balanus amphitrite* were spherical with a diameter of 50 to 60 μ, and the cytoplasm was filled with yolk granules staining bright red with Azan. Immature oocytes were observed in various stages of development having a finely granulated cytoplasm which stained yellow with Azan. The oogonia were seen as small, closely packed cells frequently in mitosis.

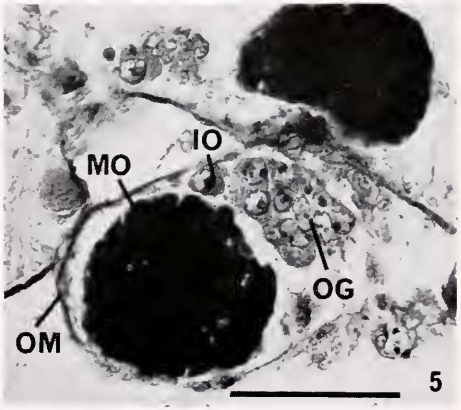
The culture media (I-XIX) were divided into four groups (A-D) each with media approximately equal in quality. Medium X did not contain ovarioles in the free floating explants cultured for seven days and this medium was therefore excluded from the comparison. Group **A** consists of four media (III, VI, V, IV) found best for maintaining ovarian cells *in vitro*. In these media no significant difference was found between the histology of uncultured tissue and explants cultured for three days (Fig. 2). On the seventh day of culture the











FIGURES 1–3. Ovarioles of *Balanus amphitrite* (Azan stain). Figure 1 shows uncultured tissue (Section 5 μ); Figure 2 shows explant cultured for 3 days (medium VI, section 8 μ); Figure 3 shows explant cultured for 7 days (medium III, section 5 μ); OM—ovariole mem-

mature oocytes were still spherical and the yolk droplets unchanged (Fig. 3). The diameter of the oocytes was normal (medium VI and V), slightly enlarged (medium III) or reduced (medium IV). Small immature oocytes adjacent to the oogonia had a normal appearance. Immature oocytes in later stages of development had a cytoplasm looser in texture than normal. The oogonia seemed normal and were in frequent mitoses. In media of group **B** (VIII, IX, XII, XVIII) the mature oocytes were deformed and the immature oocytes were enlarged already on the third day of culture. The yolk granules were unchanged in number and stainability on the third as well as on the seventh day. No clear mitosis was observed in any of the cultures. In media of group **C** (XI, XIII, XIV, XVII) the oocytes were deformed and a decrease in number of yolk granules was seen on the third day of culture. The stainability of the granules with Azan was reduced in medium XI and XVII, but remained unchanged in medium XIV. After seven days of culture the ovarian cells seemed to be in a poor condition. Still, however, the yolk granules in medium XIV and XIII had a normal appearance. In media of group **D** (I, II, VII, XV, XVI) a degeneration of most of the ovarian cells could be observed already on the third day of culture. On the seventh day the yolk granules of the mature oocytes had disappeared and the nuclei were pycnotic. The immature oocytes and oogonia were partly or completely degenerated in these media.

In some of the media free floating explants were studied histologically later than on the seventh day of culture. In medium VI, the mature oocytes on the 12th day of culture were slightly deformed but with normal yolk granules. The immature oocytes had cytoplasm of loose texture. The oogonia appeared normal, but no mitosis was observed. On the 16th day in this medium most of the yolk granules of the mature oocytes had disintegrated and the cytoplasm of the immature oocytes was partly degenerated. In cultures with media of group **B**, **C** and **D** the degeneration of the ovarian cells observed on the 7th day of culture had progressed further on the 12th and the 16th day.

Parenchymatous cells located between the ovarioles were not observed in mitosis, neither *in vivo* nor *in vitro*. In free floating explants these cells kept their structure and fishnet-like organization with no apparent increase in cell number (Fig. 2). Explants in medium VI had parenchymatous cells of normal appearance after 25 days of culture.

Cement glands in explants cultured in media of group **A** and **B** had normal appearance after three and seven days of culture: The glands had normal size, the cytoplasm was stained yellow by Azan (Fig. 6), and the nucleus contained a high amount of DNA (Fig. 7). In media of group **C** and **D** the cement glands had normal size, but the cytoplasm had a texture looser than normal. The nucelus was enlarged, but the chromatin seemed normal.

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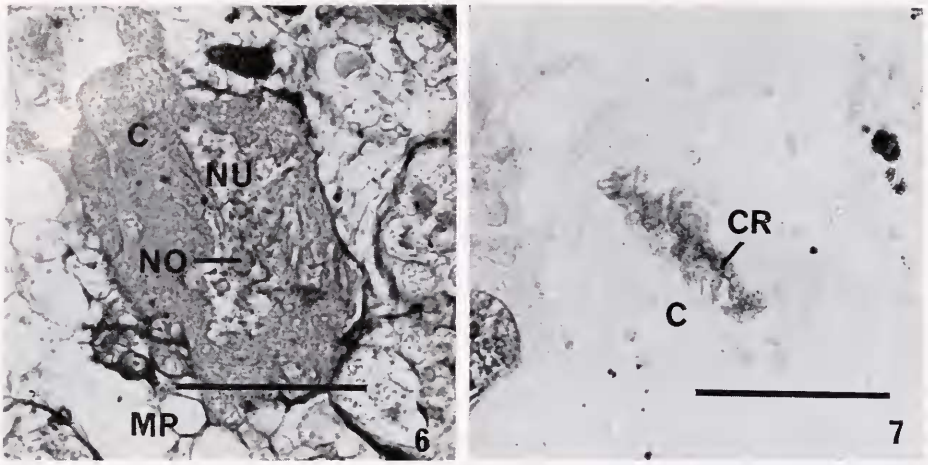
brane, MO—mature oocytes, IO—immature oocyte, OG—oogonia, MP—mantle parenchyma; marker is 50 μ.

FIGURE 4. Explant of *Balanus eburneus* cultured for 3 days (medium III, section 8 μ, Azan stain); OM—ovariole membrane, IO—immature oocyte; marker is 50 μ.

FIGURE 5. Explant of *Balanus improvisus* cultured for 3 days (medium III, section 8 μ, Azan stain); OM—ovariole membrane, MO—mature oocyte, IO—immature oocyte, OG—oogonia; marker is 50 μ.



FIGURES 6–7. Cement glands of *Balanus amphitrite* cultured for 7 days (medium III, section 8 μ). Figure 6, Azan stain; Figure 7, Feulgen reaction and Fast green; C—cytoplasm, NU—nucleus, NO—nucleolus, CR—chromatin, MP—mantle parenchyma; marker is 50 μ.

Histochemical tests for proteins, carbohydrates, and nucleic acids were applied to sections of uncultured tissues and of some of the explants. In uncultured material the yolk granules of mature oocytes showed positive reactions for proteins and protein bound SS- and SH-groups, but showed negative reactions for nucleic acids and acid mucopolysaccharides. The cytoplasm of immature oocytes was slightly positive for proteins, strongly positive for RNA (methyl-green-pyronin Y) and was stained dark blue by Toluidine blue. By treatment with ribonuclease the cytoplasm was negative for Toluidine blue, indicating that the blue staining was due to ribose of the RNA. The cytoplasm of cement glands showed negative reactions in the protein tests, but was strongly positive for RNA and stained dark blue with Toluidine blue. The latter test was negative after treatment with ribonuclease.

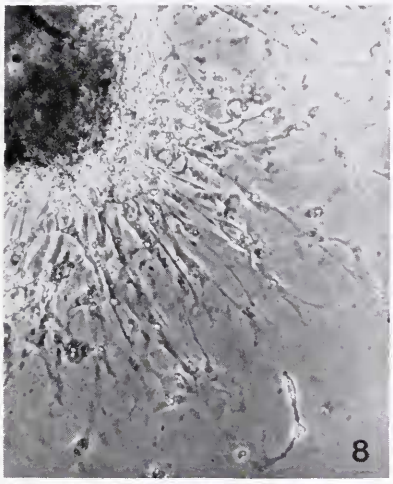
In explants cultured in media of group **A**, **B** and **C** having mature oocytes with intact yolk granules the histochemical tests showed that the granules kept their normal reactions regardless of number of yolk granules, and of size and shape of the oocytes. When the granules were partly disintegrated (medium XI, XVII and XVI) they showed slight basophilic reactions. Immature oocytes having a normal appearance after three days of culture (media of group **A**) showed normal reactions in the histochemical tests. With decreasing quality of the immature oocytes, the basophilia of the cytoplasm decreased. Cement glands cultured in media of group **A** and **B** showed normal histochemical reactions although the basophilia of the cytoplasm was somewhat lower on the 7th day.

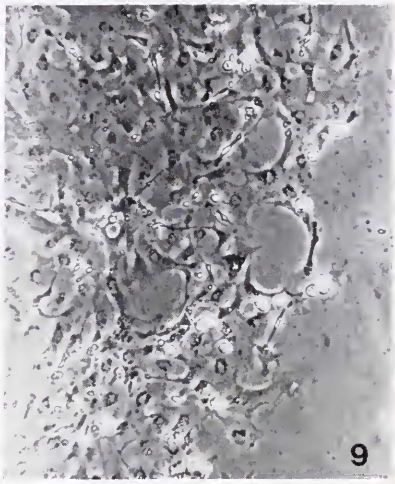
Cultures of mantle tissues of *B. improvisus* and molt staged *B. eburneus* were obtained in various media. Free floating explants were fixed after 3 and 7 days of culture for histological study. The results from these cultures were similar to those obtained for *B. amphitrite* and did not modify the ranking of the culture media for organ cultures. Media of group **A** gave better maintenance of ovarian cells from *B. eburneus* (medium III, Fig. 4) and from *B. improvisus* (medium IV,

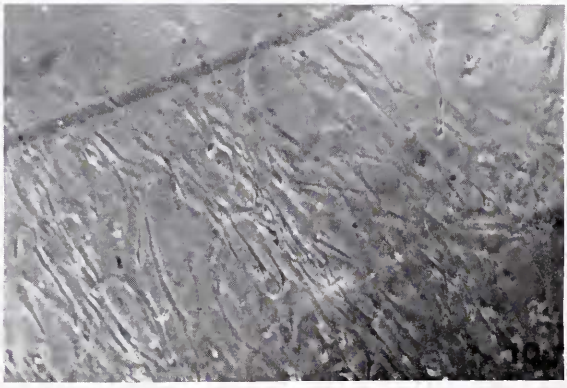
Fig. 5) than media of group **B** (*B. eburneus:* medium IX, XII, XVIII; *B. improvisus:* medium VIII), group **C** (*B. eburneus:* medium XIII), and group **D** (*B. eburneus:* medium I, XV). No difference was evident between cultures from specimens of *B. eburneus* in interecdysis (11 cultures) and proecdysis (6 cultures).

Cell outgrowth

In each culture 2 to 3 of the explants were attached to the surface of the culture flask. Within the first 48 hours of culture, cells had migrated from most of the attached explants. The migrating cells adhered to the surface. Cells were never observed to migrate from free floating explants, and no cells were seen free in the medium. Cells migrated from explants consisting of both parenchyma and ovarioles, or from explants without ovarioles, but not from explants consisting of ovarioles alone. The migrating cells represented two cell types: epithelial-like







FIGURES 8–10. Cell cultures of *Balanus amphitrite* (phase contrast microscopy). Figure 8 shows epithelial-like cells on the 3rd day of culture (medium III); Figure 9 shows epithelial-like cells on the 5th day of culture (medium III); Figure 10 shows fibroblast-like cells on the 9th day of culture (medium IX).

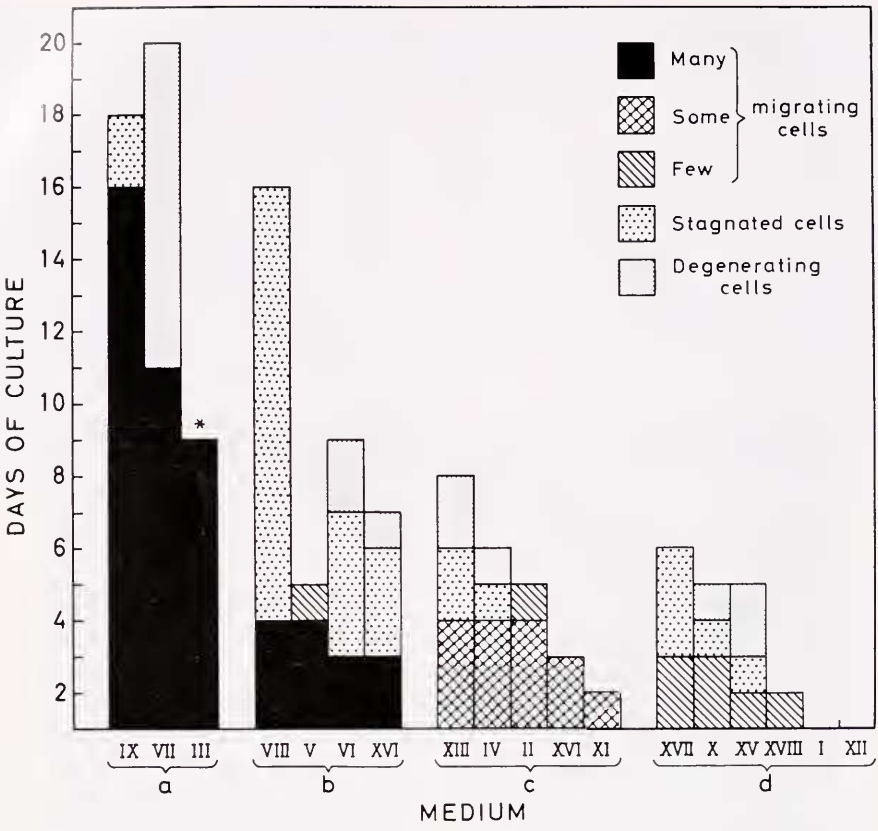
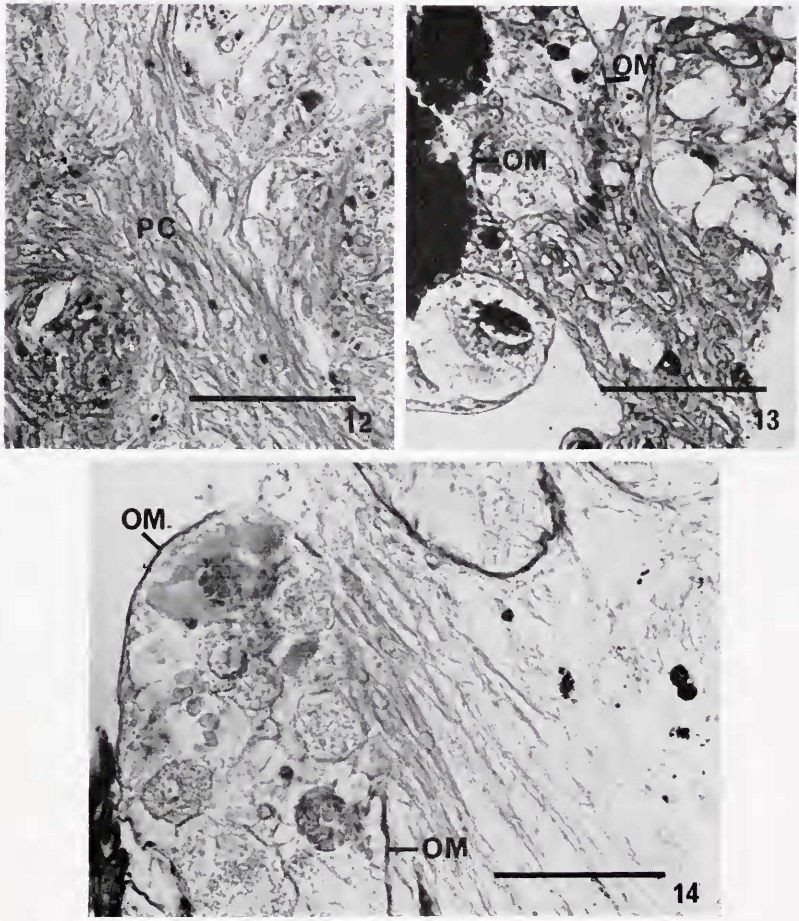


FIGURE 11. Cell outgrowth from explants of mantle tissue of *Balanus amphitrite*. The culture media are ranked according to the amount, quality and duration of migrating cells obtained in each medium. The most successful culture in each medium is illustrated.

cells which were polygonal with granules surrounding the nucleus (Fig. 8) and fibroblast-like cells which were more or less elongated and spindle shaped (Fig. 10). The epithelial-like cells were the first to migrate from the explant. Migrating cells generally had hyaline cytoplasm and mitoses were frequently observed in good cultures. After 2 to 16 days, depending upon the culture medium, the migration of cells and the frequency of cell divisions tapered off. The cells then increased in size and vacuoles and granules were observed in the cytoplasm. This stagnated phase could last for 10 days when a degeneration or cell death became evident: the vacuoles and granules in the cytoplasm became dominant, the cells became fringed and their attachment to the plastic surface gradually loosened. This phase of obvious degeneration varied from $\frac{1}{2}$ to 9 days and terminated by the disappearance of the outgrown cells.

The number, quality, and life span of the migrating cells clearly depended upon the culture medium (Fig. 11). The media in this figure are ranked according to the success of the cell outgrowth obtained in each medium and are divided into

four groups (a-d) consisting of media approximately equal in quality. In the three media comprising group a (medium IX, VII, and III) numerous cells migrated from the explants. Epithelial-like cells were most abundant during the first 7 to 9 days (Figs. 8 and 9). From then on the mitotic activity in the cultures increased and fibroblast-like cells became the far most abundant cell type (Fig. 10). The migrating cells had good appearance with hyaline cytoplasm and high mitotic activity up to the 17th day of culture. The cultures in medium IX and VII showed a sharp transition from an active proliferating phase to stagnation or degeneration, and the outgrown cells disappeared after 18 to 20 days of culture (Fig. 11). The culture in medium III, which is illustrated in Figure 11,



FIGURES 12–14. Explants being source of migrating cells (Sections 8 μ, Azan stain). Figure 12 shows parallel cells in the parenchyma (PC) (*Balanus eburneus*, 7th day of culture, medium IX); Figure 13 shows ovariole membrane (OM) partly broken (*Balanus amphitrite*, 5th day of culture, medium V); Figure 14 shows ovariole membrane (OM) partly broken (*Balanus eburneus*, 7th day of culture, medium IX); marker is 50 μ.

was terminated on the 10th day. Three other cultures with this medium (plus five cultures of *B. eburneus*) were terminated after 6 to 7 days while still in a proliferating phase with a heavy outgrowth of cells. Probably, therefore, proliferation may be obtained in medium III for a longer period than the nine days illustrated. Group b is composed of four media in which numerous cells migrated from the explants for 3 to 4 days. The cells had normal appearance with some mitotic activity, but soon disappeared or went into a stagnated phase. In group c five media are included in which some cells migrated from the explants during the first 2 to 5 days of culture. The cells generally had normal appearance, but no mitoses were observed. In media of group d only a very sparse migration of cells or none at all (medium I and XII) was obtained. The cells were irregular and fringed with heavily granulated and vacuolized cytoplasm, and no mitoses were observed.

Seven cultures with attached explants were obtained from mantle tissues of *B. improvisus* and 21 of molt staged *B. eburneus*. In the media of group a heavy cell migration was obtained in cultures of *B. eburneus* (8 cultures) and of *B. improvisus* (2 cultures) for 7 days when the cultures were terminated. In media of group b (VIII and VI) three cultures of *B. improvisus* showed some migrating cells for 3 to 4 days; in media of group c (XIII, IV, and XVI) two cultures of each species showed some migrating cells for 3 to 4 days; in media of group d (X, XV, XVIII, I and XII) eleven cultures of *B. eburneus* did not result in any cell migration. No differences were evident between cultures from specimens of *B. eburneus* in interecydsis (14 cultures) and proecdysis (7 cultures).

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The histology of attached explants was studied in cultures of *B. amphitrite* and *B. eburneus* to investigate the origin of the outgrown cells. Explants attached to the surface of the culture flasks (medium V and IX) and surrounded by migrating cells were scraped off, fixed and studied. In the *in vivo* condition the ovarioles were bounded by a continuous cellular membrane, and the parenchyma between the ovarioles had a fish-net appearance (Fig. 1). In free floating explants these conditions remained unchanged (Figs. 2–6). In attached explants the cells in the parenchyma were orientated parallel to each other (Fig. 12) while the cellular membrane of the ovariole usually was kept intact. In two cases the ovariole membrane was partly broken and an outgrowth of ovarian cells seemed probable (Figs. 13 and 14).

Discussion

The saline of Hoyle and Smyth (1963) has been successfully used in physiological experiments on barnacles (Hoyle and Smyth, 1963; McLaughlin and Hinke,

1966; Gwilliam, 1968) suggesting that the saline electrolytically is compatible with the requirements of cirriped tissues. The results of cultures with pure saline as a medium in the present study showed that cell maintenance for more than a few days as well as cell migration and multiplication depend upon exogenous sources of nuritional support besides the compatible ionic environment. D-glucose in a concentration of 0.1% did not improve the saline as a culture medium (medium II versus I) and neither did it improve a more complex medium (medium IV versus V). In cultures of gonads of the amphipod *Orchestia gamarella* Berreur-Bonnenfant (1962) found a culture medium of sea water with 0.5% glucose (plus agar as a solidifier) to be inferior to the same medium enriched with chicken embryo extract. Gonad survival was 4 days and 35 to 40 days in the respective media. Vago and Quiot (1969) reported 0.1% glucose to be included in a complex medium for cell cultures of embryonic cells of the crayfish *Astacus pallipes*. The effect of glucose on the cell cultures, however, was not investigated.

Bovine embryo extract and yeastolate in concentrations of 1% and 0.1% respectively, were found to be very good additions to the media for organ cultures as well as for cell outgrowth. Bovine embryo extract and yeastolate are complex additions and probably served as source of vitamins, amino acids, proteins, and carbohydrates. Higher concentrations of bovine embryo extract as well as addition of other tissue extracts, could further improve the media for barnacle tissue cultures.

The addition of serum had a different effect on cell maintenance and on cell migration. The four best media for organ cultures (group **A**) are without serum. For cell outgrowth the addition of 10% bovine serum or *Callinectes* hemolymph had a positive effect (medium IX versus medium III, medium VII versus IV). Chicken serum, however, seemed to have a negative effect on cell migration (medium XII versus II). Bovine serum was a more favorable addition for cell outgrowth than *Callinectes* hemolymph (medium IX versus VIII). The *Callinectes* hemolymph was pooled from animals in interecdysis. Hemolymph of crabs in other molt stages may be a more potent growth stimulator. The positive effect of serum on barnacle cell migration and proliferation is in accordance with the assumed effects of serum in control of cell multiplication in vertebrate cell cultures (Temin, Pierson and Dulak, 1972). Grace (1968) found the growth of cells in insect cell cultures to increase with hemolymph concentrations up to a certain limit when inhibitory effects appeared. For barnacle cell outgrowth more than 10% of serum had a negative effect (medium XI and X versus medium IX and VII, Fig. 11). In decapod crustaceans Eagle minimum medium and medium 199 have been successfully used in cell cultures (Vago and Quiot, 1969) and in organ cultures (Oyama and Kamemoto, 1970; Skinner and Beattie, 1971). For the barnacle cultures, however, the addition of these media did not improve the culture media.

In organ cultures loss of cells from explants is a difficulty often encountered but did not represent a problem in the present cultures. The proteid yolk granules showed high capacity for maintenance. Even under conditions when the oocytes and other ovariole cells were in an advanced state of degeneration, the yolk granules had a normal appearance. Mature oocytes were maintained for at least seven days in cultures with the four best media. In organ cultures of crab ovaries mature oocytes did not survive (Oyama and Kamemoto, 1970). In the barnacle cultures the mitotic activity of oogonia was maintained and the small immature oocytes

adjacent to the oogonia seemed normal. Larger immature oocytes, however, showed an incipient degeneration on the seventh day suggesting that even the best media could not support the previtellogenetic development required for yolk deposition. Immature oocytes in a late stage of development did neither survive in crab ovary cultures (Oyama and Kamemoto, 1970) while small immature oocytes and oogonia were maintained.

Extensive cell outgrowth with high mitotic activity was obtained from attached explants for 18 days in medium IX. At the onset of stagnation the outgrown cells formed continuous sheets surrounding each explant. Subculturing at an earlier stage may prolong the cultures in this medium. In medium VII heavy cell proliferation was obtained for 12 to 13 days. This medium was the poorest one tested for maintenance of ovarian cells in organ cultures. Parenchymatous cells, however, had a normal appearance for at least seven days. Medium VII thus seems to support cell migration and proliferation, but cannot support the maintenance of specialized ovarian cells in culture. Medium III seems to be suitable for organ cultures as well as for cell outgrowth. It was the second best medium for cell maintenance and one of the best for cell outgrowth. Tissue cultures as obtained by the present technique should allow investigations with physiological, endocrinological, and pathological aspects of barnacle cells *in vitro*.

Ovarian tissue is generally a fruitful source of migrating cells in primary cultures. The origin of migrating cells from such complex organs as the ovary is, however, difficult to determine (Vaughn, 1971). In the barnacle cultures the explants included unspecialized parenchymatous cells and/or ovarian cells in various stages of development and specialization. Migrating cells were possibly of non-ovarian origin since ovarioles in explants being donors of migrating cells usually were kept intact, the parenchymatous cells in donor explants had a paraellel orientation (Fig. 12), and cell migration was never observed from isolated ovarioles. In two cases, however, the ovariole membrane was partly broken and an outgrowth of ovarian cells seemed probable (Figs. 13 and 14). In insect ovarian cultures Jones and Cunningham (1960) assumed the boundary membrane of the follicular cells to be a barrier for the penetration of these cells into the cellular field, and Grace (1958) found no cell migration from naked ovaries. Stanley and Vaughn (1968) circumvented the problem by using minced ovaries. Dispersion of the cells in barnacle explants at the start of the cultures may increase the ovarian part of the cell donation. With the general high proliferation potential of ovarian cells and the frequent cell divisions obtained in germinal cells in the present cultures, this may increase the lifespan and growth capacity of barnacle cells *in vitro*.

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Summary

1. Various components were tested as nutrients in media for barnacle tissue cultures. Bovine embryo extract and yeastolate were favorable additions for both cell outgrowth and organ cultures. Glucose did not improve the media. Bovine

serum and *Callinectes* hemolymph had a negative effect. Mixed sera in concentrations higher than 10% were negative for both types of cultures. Addition of Minimum Medium Eagle and Medium 199 did not improve the barnacle culture media.

2. In organ cultures mantle parenchyma were obtained for 25 days, ovarioles and cement glands for at least 7 days with no change in structure and organization. Mature oocytes and young immature oocytes were maintained for at least 7 days, and oogonia kept a high mitotic activity. Immature oocytes in later stages of development were not maintained.

3. Extensive cell outgrowth forming large surface spreads and with cells showing high mitotic activity were obtained from attached explants for 18 days. The initial cell migration consisted of epithelial-like cells, later, fibroblast-like cells were most abundant. A predominantly non-ovarian origin of the migrating cells seemed probable. It is suggested that the ovarian part of cell donation may be increased by early dispersion of the cells in the explants.

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